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Inhibitory mechanisms of glabridin on tyrosinase

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ABSTRACT

Tyrosinase is an oxidase that is the rate-limiting enzyme for controlling the production of melanin in the human body. Overproduction of melanin could lead to a variety of skin disorders. Glabridin, an isoflavan, isolated from the root of *Glycyrrhiza glabra* Linn, has exhibited several pharmacological activities, including excellent inhibitory effects on tyrosinase. In this paper, the inhibitory kinetics of glabridin on tyrosinase and their binding mechanisms were determined using spectroscopic, zebrafish model and molecular docking techniques. The results indicate that glabridin reversibly inhibits tyrosinase in a noncompetitive manner through a multiphase kinetic process with the IC_{50} of 0.43 μmol/L. It has been shown that glabridin had a strong ability to quench the intrinsic fluorescence of tyrosinase mainly through a static quenching procedure, suggesting a stable glabridin-tyrosinase complex may be generated. The results of molecular docking suggest that glabridin did not directly bind to the active site of tyrosinase. Moreover, according to the results of zebrafish model system, glabridin shows no effects on melanin synthesis in zebrafish but presents toxicity to zebrafish embryo. The possible inhibitory mechanisms, which will help to design and search for tyrosinase inhibitors especially for glabridin analogues, were proposed.
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1. Introduction

Tyrosinase (EC 1.14.18.1), a multifunctional copper-containing oxidase, is common in mammals, insects, plants and microorganisms [1,2]. Tyrosinase plays a crucial role in melanin biosynthesis pathway that is responsible for melanogenesis of animals [3]. And it actually catalyzes the hydroxylation of tyrosine to L-DOPA (L-3, 4-dihydroxyphenylalanine) and the subsequent oxidation of L-DOPA to DOPA quinone [4,5] which transforms into melanin and other polyphenolic compounds after a series of spontaneous chemical reactions [6]. Overproduction of melanin may lead to a variety of skin diseases, such as melasma, age spots and freckles [7,8]. Tyrosinase inhibitors, such as kojic acid, N-Phenylthiourea, arbutin or hydroquinone, are used for the treatment of these diseases [9]. However, these inhibitors cause side effects like dermatitis, skin irritation, DNA damage and cancers [10–12]. Therefore, searching for tyrosinase inhibitors with higher bioactivity and lower toxicity has become a hot research topic.

Glabridin (Fig. 1), an isoflavan, was firstly isolated from the root of *Glycyrrhiza glabra* Linne in 1976 [13]. It has been extensively studied and has a variety of pharmacological activities, such as prevention of low density lipoprotein oxidation [14], anti-inflammatory properties [15], neuroprotective effects [16], estrogenic properties [17] and regulation of energy expenditure and metabolism [18]. In addition, it also has some less frequently reported biological activities like antimicrobial activity [19], anti-cancer properties [20] and inhibition of melanogenesis [21]. Glabridin has been demonstrated to inhibit the activity of tyrosinase in B16 murine melanoma cells [21]. It also displayed a better tyrosinase inhibition than the widely used kojic acid in enzymatic assays [22]. As we all known, safety is a primary consideration for tyrosinase inhibitors. It has been demonstrated that glabridin is non-cytotoxic to G361 human melanocyte [23], but the safety data of glabridin on multicellular organisms is still lacking. Although the inhibitory efficacy of glabridin on tyrosinase was evaluated, the molecular mechanisms underlying these interactions are still unclear. Moreover, glabridin is susceptible to oxidation degradation, which will result in easy loss of pharmacological activities [24], so it is necessary to search tyrosinase inhibitors with better stability [25]. Due to the strong inhibitory effects of glabridin on tyrosinase, it would be a shortcut to screen glabridin analogues as potential tyrosinase inhibitors. Therefore, it is important to find out the structure-function relationship of glabridin on the inhibitory effects of tyrosinase.

In this study, the inhibitory efficacy of glabridin on tyrosinase was completely investigated by both in vitro assay using mushroom tyrosinase and in vivo test using zebrafish model which is also applied to evaluating the safety of glabridin. The molecular mechanisms underlying this interaction were extensively explored using fluorescence quenching method and molecular docking techniques. All the results

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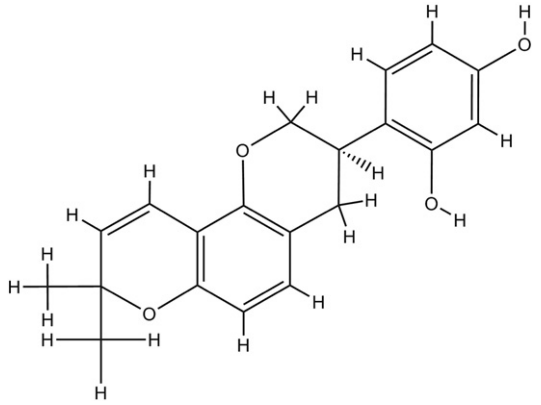

Fig. 1. Structure of glabridin.

will be helpful for the exploration of tyrosinase inhibitors, especially for glabridin analogues, with better stability, higher bioactivity and lower toxicity and will provide fundamental data for the pharmacological and physiological activities of glabridin.

2. Materials and methods

2.1. Materials

Glabridin (98.5%) was bought from Push Bio-technology (Chengdu, China). L-tyrosine, L-DOPA and mushroom tyrosinase (EC 1.14.18.1) were bought from Sigma-Aldrich (St. Louis, MO, USA). 1-phenyl-2-thiourea (PTU), kojic acid and arbutin were obtained from Aladdin Bio-technology (Shanghai, China). All other chemicals used were of analytical grade.

2.2. Tyrosinase activity assays

The assays were carried out as previously reported with several modifications [26]. Glabridin was dissolved in dimethyl sulphoxide at 10 mmol/L and diluted for dose dependence in vitro experiments. L-tyrosine was used as a substrate for monophenolase activity assays and L-DOPA was used as a substrate for diphenolase activity assays. The activity assays used 3 mL of reaction medium containing 0.5 mM L-tyrosine or 0.5 mM L-DOPA in 50 mM Na_2HPO_4–NaH_2PO_4 buffer (pH 6.8). The final concentrations of tyrosinase were 33.33 U/mL for monophenolase activity assays and 13.33 U/mL for diphenolase activity assays. The reactions were performed at 298 K and pH = 6.8. The optical densities of the samples were then determined at 475 nm using Ultraviolet-Visible spectrophotometer (UV 2550, Shimadzu). For monophenolase activity assays, the absorbance at 475 nm was measured every 30 s in the first 2 min and then every 60 s. Kojic acid was used as a positive control. Triplicate absorbance readings of positive controls and test samples were averaged, respectively. The relative enzymatic activity tested without glabridin was defined as 100%. Relative activity (%) = (slope of reaction kinetics equation obtained by reaction with the inhibitor) / (slope of reaction kinetics equation obtained by reaction without the inhibitor) × 100%.

2.3. Kinetic analysis for noncompetitive-type inhibition

The noncompetitive inhibition mechanisms can be described by the Lineweaver–Burk equation in double reciprocal form:

$$\frac{1}{v} = \frac{K_m}{V_{max}}\left(1 + \frac{[I]}{K_i}\right)\frac{1}{[S]} + \frac{1 + \frac{[I]}{K_i}}{V_{max}} \quad (1)$$

and secondary plot can be constructed from

$$In. = \frac{[I]}{K_i V_{max}} + \frac{1}{V_{max}} \quad (2)$$

where v is the enzyme reaction rate in the absence and presence of glabridin. K_i and K_m are the inhibition constant and Michaelis–Menten constant, respectively. Their values can be obtained from the above equations. [I] and [S] are the concentrations of an inhibitor and a substrate, respectively. In. is the intercept value of the Lineweaver–Burk curve. The secondary plot of In. vs. [I] is linearly fitted, then K_i can be calculated.

2.4. Fluorescence quenching

Fluorescence spectra were recorded using F-4600 spectrofluorophotometer (Hitachi Inc., Japan) equipped with a 150 W xenon lamp and a thermostat bath. A 2.5 mL solution containing 400 U/mL tyrosinase was added to the quartz cuvette, and then titrated by successive addition of 1 mmol/L glabridin solution using a micropipette (to give a concentration range from 0 to 137.93 μmol/L). These solutions were allowed to stand for 5 min in the thermostat bath to equilibrate, and then the fluorescence emission spectra were measured at wavelengths of 290–500 nm upon excitation at 280 nm. The excitation and emission bandwidths were both 5 nm. The appropriate blanks corresponding to the sodium phosphate buffer were subtracted to correct for background fluorescence.

2.5. Molecular docking studies

The docking program AutoDock (4.2.6) was utilized to explore the probable interactions between compounds and tyrosinase [27]. The 3D structures of compounds were generated in Chem3D Ultra 8.0, and the X-ray crystal structure of *Agaricus bisporus* tyrosinase (PDB ID: 2Y9X) was retrieved from the RCSB Protein Data Bank (http://www.rcsb.org/pdb) [28]. AutoDockTools 1.5.4 package was used for preparing all input files, and charge of +2 was assigned to each copper ion. To carry out docking simulations, a grid box was defined to enclose the active site with dimensions of 88 Å × 76 Å × 106 Å and a grid spacing of 0.375 Å. The grid maps for energy scoring were calculated using AutoGrid. Docking calculations were performed using the Lamarckian Genetic Algorithm and the search parameters were set to 100 times. The best docking result was chosen according to the lowest total score. The figures were produced by using the PyMOL molecular graphics system (http://www.pymol.org).

2.6. Effects on melanin synthesis in zebrafish

The effects on melanin synthesis in a zebrafish model system were assayed according to the method described by Choi et al. [29]. Adult zebrafish were obtained from China Zebrafish Resource Center and kept in acrylic tanks with 14/10 h light/dark cycle at 301 K. Embryos were obtained from natural spawning that was induced in the morning by turning on the light. Embryos were collected within 30 min and arrayed by pipette, 10–15 embryos per well, in 24-well plates. Test compounds, including arbutin, kojic acid and glabridin, were dissolved in the embryo medium with or without 1% DMSO and then added to plates by using pipette from 9 to 57 hpf (hours post fertilization). In all experiments, 0.25 mmol/L PTU was used to generate transparent zebrafish and considered as a standard positive control. The effects on the pigmentation of zebrafish were observed under the microscope at 57 hpf.

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